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(71) Applicants *(for all designated States except US)*: KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY [KR/KR]; 39–1 Hawolkok–dong, Sungbuk–ku, 136–130 Seoul (KR). HAITAI CONFECTIONERY CO., LTD. [KR/KR]; 108–2 Yangpyung–dong–5–ga, Youngdeungpo–ku, 150–105 Seoul (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CHOI, Eui–Sung [KR/KR]; #102–507 Dasol Apt., 395–3 Kung–dong, Yusong–ku, 305–335 Taejon–si (KR). RHEE, Sang–Ki [KR/KR]; #Ka–101, Keukdong Villa, Kwangjang–dong, Kwangjin–ku, 143–210 Seoul (KR). SOHN, Jung–Hoon [KR/KR]; #103–506 Noori Apt., Wolpyong–dong, Seo–ku, 302–280 Taejon–si (KR). PARK, Soo–Dong [KR/KR]; #109–1305 Hana Apt., 153 Shinsung–dong, Yusong–ku, 305–345 Taejon–si (KR). LEE, Yoon, Hyoung [KR/KR]; #933–1503 Myohyang Apt., Sanbon–dong, Kunpo–si, 435–040 Kyoungki–do (KR). LEE, Seung, Jae [KR/KR]; #7–205 Pucheon Apt., 566–1 Simgokbon–dong, Sosa–ku, Pucheon–si, 422–240 Kyoungki–do (KR). JANG, Jae, Kweon [KR/KR]; #102 Jaewon Villa, 229–8 Seokchon–dong, Songpa–ku, 138–190 Seoul (KR). CHOI, Seok, Keun [KR/KR]; #503 Jinro Apt., 10 Myunmok–dong, Chungrang–ku, 131–208 Seoul (KR). SON, Young, Rok [KR/KR]; #919 Nasanmisi 860 Officetel, 13–3 Kaepo–dong, Kangnam–ku, 135–240 Seoul (KR).

(74) Agent: LEE, Won–Hee; Suite 805, Sung–ji Heights II, 642–16 Yoksam–dong, Kangnam–ku, 135–080 Seoul (KR).

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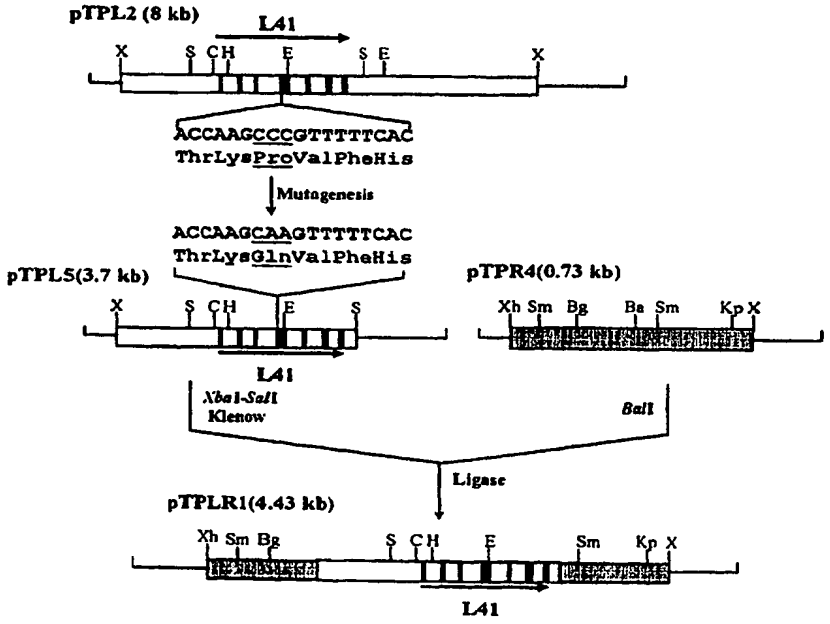

(57) Abstract

The present invention relates to a transforming vector and a process of transformation thereby, more specifically to a transforming vector comprising a cycloheximide–resistant gene and a ribosomal DNA. The transforming vector and the transforming process thereby is applicable to the efficient and stable integration of desired DNA into yeast genome, thus providing useful tools for the production of a natural pignent, astaxanthin.

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VECTOR FOR THE TRANSFORMATION OF *Phaffia rhodozyma* AND PROCESS OF TRASFORMATION THEREBY

FIELD OF THE INVENTION

The present invention relates to novel vectors for the transformation of *Phaffia rhodozyma* and to a process of transformation thereby. Particularly, this invention relates to an L41 gene encoding a ribosomal protein derived from *Phaffia rhodozyma* which is useful for producing natural pigment astaxanthin; an L41 gene mutated to a cycloheximide-resistant form; a *Phaffia rhodozyma* ribosomal DNA; a vector for the stable transformation of *Phaffia rhodozyma*, comprising said mutated L41 gene and said ribosomal DNA; and a process of transformation thereby.

BACKGROUND

Phaffia rhodozyma is reddish yeast species producing astaxanthin, the useful natural pigment. Astaxanthin is a member of the carotenoids, which are represented by β-carotene, the precursor of vitamin A. Naturally, astaxanthin is widely distributed, especially to Crustacea, trout and salmon as their main pigment, although they cannot synthesize astaxanthin and should be supplied with it from the diet. Thus, it has been considered necessary to add the pigment in the

cultivation of Crustacea, trout and salmon, so that the added pigments to the Crustacea and fishes may attract the consumers and give better flavors. This carotenoid pigment plays key roles in the physiological metabolism of human as well as animals, with known effects such as the precursor of vitamin A, the enhancement of immunological function, the antioxidant activity, the prevention of cancer and senescence, etc.

Because of increasing interests in *Phaffia rhodozyma* and pigments produced thereby, there have been a number of reports concerned about the culture of *Phaffia rhodozyma*. However, these researches have been focused on how the inexpensive materials can be used for its culture, and have resulted in the development of culturing methods, in which various local products may be employed, such as alfalfa juice (Okagbue *et al.*, *Appl. Microbiol. Biotechnol.*, 20, 33, 1984), molasses (Haard *et al.*, *Biotechnol. Lett.*, 10, 609, 1988), the byproducts of grape juice processing (Lango *et al.*, *Biotech. Forum Europe*, 9, 565, 1992), peat hydrolyzate (Martin *et al.*, 58, 223, 1993), the byproducts of corn wet-milling (Hayman *et al.*, *J. Ind. Microbiol.*, 14, 389, 1995), and the mixture of sugar cane extract, urea and phosphoric acid (Fontana, *et al.*, *Appl. Biochem. Biotechnol.*, 57/58, 413, 1996).

Although little is known about the genetics of *Phaffia rhodozyma*, the physiological features of

Phaffia rhodozyma have been disclosed and the *Phaffia rhodozyma* mutant has recently been selected to produce higher level of the pigment (Johnson *et al.*, *Crit. Rev. Biotechnol.*, 11, 297, 1991; An *et al.*, *Appl. Environ. Microbiol.*, 55, 116, 1989; Chumpolkulwong *et al.*, *J. Ferment. Bioeng.*, 75, 375, 1997; Lewis *et al.*, *Appl. Environ. Microbiol.*, 56, 2944, 1990). In addition, a genetic analysis enlightened the ploidy and sexual cycle of *Phaffia rhodozyma*. In a flow cytometry study, Calo-Mata and Johnson found that no strain was haploid and that most were polyploid (Calo-Mata *et al.*, *Yeast Gen. Mol. Biol. Meet.*, 126, 1996). A pedogamic sexual process of conjugation has been also described (Golubev *et al.*, *Yeast*, 11, 101, 1995).

Although *Phaffia rhodozyma* is potentially useful for the production of astaxanthin and the like, the pigment level in the wild type of *Phaffia rhodozyma* is very low. Therefore, there have been increasing attempts to develop novel mutant strain of *Phaffia rhodozyma*, which can produce the higher level of the pigment. However, these attempts have been hampered by the reduced growth rate and genetic instability, which may occur when the pigment content in a mutant exceeds over the optimal range.

Another obstacle to the progress of the mutant is the method of mutagenesis. Chemical mutagenesis

procedure has been performed conventionally, but it is associated with the simultaneous mutation of undesired genes leading to pleiotropic effects such as the reduction of growth rate, the prolongation of induction time in the fermentation, etc. Furthermore, the genome of the mutant strain is not stable, since its subculture often decreases the yield of the pigment.

To solve these problems in the conventional breeding procedures and to enlarge the applicability of *Phaffia rhodozyma*, molecular breeding approaches have been initiated recently, using genetic transformation. However, since most of *Phaffia rhodozyma* strains are polyploid and thus cannot be made to be an auxotrophic variant by the method conventionally applied to yeast, preferable is the approach employing antibiotics-resistant genes as selectable marker. More recently, there was reported a transformation system in which *Phaffia rhodozyma* actin promoter and G418-resistant gene were used for the transformation of *Phaffia rhodozyma*, although it showed poor transformation efficiency (Wery et al., Gene, 184, 89, 1997).

On the other hand, cycloheximide, an eukaryote-specific antibiotics, is applicable to the selection of yeast transformants. The target molecule of cycloheximide action is aminoacyl-tRNA binding site (A site), where it blocks peptidyl transferase activity.

As a result, it inhibits protein synthesis and cell growth in eukaryotes, without an effect on the organelles such as chloroplasts and mitochondria. Furthermore, it has been found that cycloheximide interacts with ribosomal protein L41, and that a mutation in L41 gene confers cycloheximide-resistance on the yeast transformants. Thus, cycloheximide and related mutant form of L41 gene are widely applicable to the process of transformation for yeasts.

Recent studies support the applicability of L41 gene to selectable marker in yeasts. Takagi et al. found that amino acid substitution through the mutagenesis of *Saccharomyces cerevisiae* L41 gene conferred cycloheximide-resistance, suggesting the usefulness of L41 gene as a selectable marker (Takagi *et al., J. Bacteriol.*, 174, 254-262, 1992). Mutoh et al. proposed a biotechnological tool using *Candida maltosa* L41 gene as a selectable marker (Mutoh et al., *J. Bacteriol.*, 5383, 177, 1995). As it is well known that cycloheximide-resistance is conferred on *Candida utilis* as well as *Phaffia rhodozyma* by the substitution of 56th amino acid residue in the L41 protein (Keiji Kondo *et al., J. Bacteriol.*, 7171, 177, 1995), transformation system thereby has been developed. Similar approaches have been attempted in *Kluyveromyces lactis* and *Schwanniomyces occidentalis* (Dehoux *et al., Eur. J. Biochem.*, 213, 841-843, 1993; Pozo *et al., Eur.*

J. Biochem., 213, 849-857, 1993). On algae *Tetrahymena*, the resistance is conferred by substitution of 40th amino acid residue, methionine to glutamine (Roberts *et al., Exp. Cell. Res.*, 312, 81, 1973).

To overcome the foregoing and other disadvantages, we, the inventors of the present invention, have noted that cycloheximide and related mutation in L41 gene may be used to develop an efficient transformation system, in which a foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and in which the transformants are undoubtedly selected. To develop such system, we have constructed transforming vectors comprising the antibiotics-resistant gene and the targeting gene, which is used for the stable integration of foreign gene. We transformed *Phaffia rhodozyma* with such vectors, according to a modified method for electrotransforming *Cryptococcus neoformans*, a member of Basidiomycetes, of which *Phaffia rhodozyma* is also another member (Kim *et al., Appl. Environ. Microbiol.*, 64, 1947, 1998).

The present invention is performed by cloning and sequencing *Phaffia rhodozyma* L41 gene; modifying the L41 gene by the mutagenesis of the region responsible to cycloheximide-resistance; constructing the vectors for transforming by inserting ribosomal DNA into the mutated L41 gene; transforming *Phaffia rhodozyma* with

the vector by electroporation method; and verifying the stable integration of the vector into the genome of the transformants.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a vector for transforming *Phaffia rhodozyma* efficiently.

It is a further object of this invention to provide an antibiotics-resistant vector for transforming *Phaffia rhodozyma*, which comprises the L41 protein of *Phaffia rhodozyma*.

It is an additional object of this invention to provide a L41 gene encoding the L41 protein of *Phaffia rhodozyma*.

It is another object of this invention to provide a mutated L41 gene that can be used as a cycloheximide-resistant gene.

It is still another object of this invention to provide a ribosomal DNA of *Phaffia rhodozyma*, which can be used to enhance the integration efficiency of foreign DNA into *Phaffia rhodozyma* genomes.

It is also an object of this invention to provide a process of transforming *Phaffia rhodozyma* by electroporation.

Further objects and advantages of the present invention will appear hereinafter.

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

The present invention provides an L41 gene encoding a ribosomal protein originated from *Phaffia rhodozyma*.

In addition, this invention provides a mutated L41 gene in which the amino acid at the position 56 is replaced by glutamine. Since the amino acid residue is responsible for the cycloheximide-resistance, this mutated gene in a vector is useful for a selectable marker.

This invention also provides a ribosomal DNA derived from *Phaffia rhodozyma*.

In addition, this invention provides a vector comprising a cycloheximide-resistant gene and a ribosomal DNA derived from *Phaffia rhodozyma*.

In such aspect of this invention, also provided is a vector, pTPLR1 comprising the mutated L41 gene of *Phaffia rhodozyma* and a portion of the *Phaffia rhodozyma* ribosomal DNA.

This invention also provides a process of transforming *Phaffia rhodozyma* with the vector by electroporation.

In such aspect of this invention, the vector is preferably cleaved into a linear form, and the preferable condition for electroporation is such that

electric pulse is 0.8~1.2 kV, an internal resistance is 400~800 Ω, and a capacitance is 25~50 μF.

Further features of the present invention will appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is nucleotide and deduced amino acid sequences of L41 gene encoding *Phaffia rhodozyma* ribosomal protein, where

Open boxes: TATA and CAAT sequences;

Underlined: the position of primers;

Bold letters: consensus sequence in splicing region of intron;

Open circle: amino acid residue at position 56

Figure 2 represents the construction of pTPLR1 vector and its restriction map, where

Numbers in parentheses: the sizes of inserts;

Blank boxes: DNA fragment containing L41 gene;

Grey boxes: rDNA fragments;

Black boxes: exons of L41 gene;

Thin lines: pBluescript SK(+) sequence;

Horizontal arrow: transcriptional direction of L41 gene;

X: *Xba*I site; S: *Sal*I site; C: *Cla*I site;

H: *Hind*III site; E: *Eco*RI site; Xh: *Xho*I site;

Sm: *Sma*I site; Bg: *Bgl*I site; Ba: *Bal*I site;
Kp: *Kpn*I site;

Figure 3 represents the restriction map of pTPLR1, the vector of this invention,

Figure 4 represents the relationship between the condition of electroporation and the transformation efficiency or cell viability;

Figure 5 represents Southern blot analysis of pTPLR1 transformants, where

C: nontransformant control;
1 to 5: pTPLR1 transformants;

Figure 6 represents schematically the mode of pTPLR1 integrated into the chromosome.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is based upon the notion that cycloheximide and related mutation in L41 gene may be used to develop a transformation system, in which foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and in which the transformants are undoubtedly selected.

Hereinafter, the present invention is described in detail.

In one aspect, the present invention provides a L41 gene encoding a *Phaffia* ribosomal protein.

In a preferred embodiment, we have obtained genomic and cDNA sequences containing the L41 gene encoding a *Phaffia rhodozyma* ribosomal protein, and these sequences are prepared from a *Phaffia rhodozyma* strain (ATTC 24230).

The L41 gene identified in this invention shows high homology with other known L41 gene of yeasts, but contains 6 introns which have specific sequences in 5′ and 3′ regions of each intron. The genomic sequence described by SEQ ID NO: 1 contains the L41 gene of 1,223 bp, which in turn contains the cDNA sequence described by SEQ ID NO: 2. Of the deduced amino acid sequence described by SEQ ID NO: 3, proline at position 56 is responsible for the sensitivity to cycloheximide (see FIG 1).

In another preferred embodiment, the cloned L41 gene is modified by site-directed mutagenesis, so that the mutated L41 gene is made to be a cycloheximide-resistant gene, or gene which can confer resistance to cycloheximide on an acceptor organism. Particularly, the mutagenesis is performed to replace the proline residue by glutamine, at the position 56 (see FIG 2).

The mutagenesis in this invention includes all the

possible modification of triplet codon in the amino acid position 56. For example, the codons for proline 56 may be replaced by all possible triplet codons for glutamine.

This invention also provides a ribosomal DNA (hereinafter "rDNA") derived from *Paffia* yeast.

In this invention, rDNA means not only a DNA sequence which is transcribed to bear all types of eukaryotic ribosomal RNA, but also a non-transcription spacer (hereinafter, "NTS"), or a DNA sequence between the transcribed rDNA. rDNA can be preferably used to enhance the integration efficiency of foreign DNA into host genomes because rDNA sequence is highly repeated as tandem units in the eukaryotic genomes.

In a preferred embodiment, we identified the rDNA which is described by SEQ ID NO: 4. This rDNA sequence contains NTS.

This invention provides a transforming vector comprising a cycloheximide-resistant gene and a rDNA.

According to one preferred embodiment, the rDNA may be used to enhance the integration efficiency of foreign DNA into the host genome.

According to another preferred embodiment, the *Phaffia rhodozyma* L41 gene modified to cycloheximide-resistant gene is employed as a selectable marker in

the transforming vector (see fIG 2). This transforming vector is useful for the stable introduction of a foreign gene into a host genome.

More particularly, this invention provides pTPLR1, a vector for transforming yeasts, most preferably for transforming *Phaffia rhodozyma*, which comprises an NTS portion of *Phaffia rhodozyma* rDNA and a mutated *Phaffia rhodozyma* L41 gene where the codon for proline at amino acid position 56 is replaced by the codon for glutamine (see FIG 3).

The transforming vectors of this invention may be readily modified and improved within the spirits and scope of this invention. For example, the transforming vector of this invention may include diverse L41 genes modified through various mutagenesis procedures and diverse rDNA sequences originated from various organisms.

In another aspect of this invention, also provided is a process of transforming yeasts with foreign DNA. The process is based upon the established method for transforming *Cryptococcus neoformans*, but optimized to yeasts, using an antibiotics-resistance gene derived from yeasts instead of the bacterium-derived counterpart.

In a preferred embodiment, the transforming vector is cleaved into a linear form before transformation.

The restriction enzymes used and the reaction may be selected carefully so that foreign DNA is efficiently introduced into host genome and only desired sequences of the vector are inserted to the host genome.

In the transforming process of this invention, an electroporation procedure is employed. According to another embodiment, the preferable condition for electroporation is such that electric pulse is 0.8~1.2 kV, an internal resistance is 400~800 Ω, and a capacitance is 25~50 μF. After electroporation, the yeast cells are cultured at 23°C for 14~16 hours, then spread on solid medium containing cycloheximide, and further cultured at 23°C for 4~5 days. Assessing the effects of various conditions for the electroporation on the cell viability and the transforming efficiency (see FIG 4) reveals that abundant transformants are produced under such condition as electric pulse of 0.8 kV, an internal resistance of 600 Ω, and a capacitance of 50 μF.

In still another embodiment, Southern blot analysis is used to verify the stable integration of foreign DNA (see FIG 5 and 6). The result confirms that the introduced genes are stably maintained in host genome, even after multiple subcultures on the medium without cycloheximide.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: The isolation of *Phaffia rhodozyma* L41 gene

To isolate genomic DNA sequence encoding *Phaffia rhodozyma* ribosomal protein L41, we synthesized two PCR (; polymerase chain reaction) primers, the sequences of which were deduced from the nucleotide sequence of other yeast L 41 genes and described by SEQ ID NO: 5 (CYH1) and SEQ ID NO: 6 (CYH3). PCR was performed in which the synthetic oligonucleotides, CYH1 and CYH3 were used as PCR primers and in which genomic DNA isolated from *Phaffia rhodozyma* (ATCC 24230) was employed as template. The PCR produced 700 bp DNA fragments containing L41 gene, which were then brought to the labeling reaction using digoxigenin (DIG)-labeling kit (Boehringer Mannheim, Germany) so as to be used as a probe for Southern blot analysis. To clone full-length L41 gene, Southern hybridization was performed as described in the work of Sambrook et al.

(Sambrook et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989) in a solution containing 5X SSC, 0.1% (w/v) sarcosyl, 0.02% (w/v) SDS, 5% blocking agent, and 50% (v/v) formamide, at 42°C. A strong hybridization signal was observed from an 8-kb *Xba*I fragment, and the *Xba*I fragments of 7 to 9-kb were isolated and ligated into pBluescript SK(+) (Stratagene, USA) to make a minilibrary. A clone (pTPL2), hybridizing with the PCR product was identified in a further Southern blot analysis in which the DNA fragments of the minilibrary were blotted onto the membrane.

To identify the L41 gene without intron, *Phaffia rhodozyma* L41 cDNA was isolated by the method of rapid amplification of cDNA ends (; RACE) with 3′-RACE (GIBCO BRL, USA) and 5′-RACE (Clontech, USA) kits. Total RNA was prepared by the method of Elion and Warner (Elion *et al., Cell*, 39, 663-673, 1984). Then mRNA was selected from the total RNA, using mRNA isolation kit (Novagen), and brought to 3′ RACE reaction in which synthetic oligonucleotide described by SEQ ID NO: 7 was used as 3′ RACE primer, and 5′ RACE reaction by SEQ ID NO: 8 as 5′ RACE primer.

The sequencing of the 3′ and 5′ RACE products suggested that a putative open reading frame of 1,223 bp be interrupted by six introns. The cloned L41 gene was found to show high homology with those of other

yeasts. However, the number of introns and their organization in the *Phaffia rhodozyma* L41 gene were quite different from the other yeast L41 genes, where there is only one intron. GTPuNGT sequence and PyAG sequence were conserved in 5′ and 3′ ends, respectively, of *Phaffia rhodozyma* L41 gene; this conserved sequences have also reported in the *Phaffia rhodozyma* actin introns. The *Phaffia rhodozyma* L41 gene encodes ribosomal protein comprising 106 amino acids, and most notably, proline at position 56 is identified to the amino acid residue responsible for the sensitivity to cycloheximide. The genomic DNA sequence of *Phaffia rhodozyma* L41 gene was registered in GenBank on May 19, 1997, with accession NO. AF 004672 (see FIG 1).

Example 2: Cycloheximide-resistant L41 gene

To confer the cycloheximide-resistance on L41 gene, we performed the site-directed mutagenesis which resulted in the amino acid converting proline 56 to glutamine. Specifically, mutagenesis was carried out with the QuickChange in vitro mutagenesis kit (Stratagene) as described in the manufacturer's instructions with complementary mutagenic primers corresponding to amino acids 52 to 59 and described by SEQ ID NO: 9 and 10. Digested from the 8.0-kb fragment in Example 1, the 2.2-kb *Sal*I fragment was replaced

with the mutated fragment.

Example 3: The isolation of ribosomal DNA

Ribosomal DNA (rDNA) in this invention was exploited to enhance the integration efficiency of foreign DNA into *Phaffia rhodozyma* genomes. To clone the rDNA fragment, two pairs of PCR primers, described by SEQ ID NO: 11, 12 (corresponding to 18S rDNA part) and 13, 14 (corresponding to 28S rDNA part), were designed from the known partial rDNA sequence of *Phaffia rhodozyma*.

By PCR with these two pairs of primers, two DNA fragments were obtained, one of which was 1.5-kb fragment containing the 5.8S rDNA NTS (; non-transcription spacer) region with the primers described by SEQ ID NO: 11 and 14, and the other of which was 6-kb fragment containing the 5S rDNA NTS region with the primers described by SEQ ID NO: 12 and 13.

Two DNA fragments were used as a probe for cloning the rDNA unit in genomic Southern blot analysis, followed by the construction of minilibrary, as described in Example 1. Multiple rounds of Southern hybridization identified an 8.5-kb *Hin*dIII fragment, which was cloned and whose identity was confirmed by partial sequencing. A 730-bp *Xho*I and *Xba*I fragment of the 8.5-kb fragment, which spans NTS region between 5S

and 18S rDNA, was subcloned in pBluescript and the resulting vector was designated as pTPR4. The sequencing of pTPR4 enlightened that the cloned rDNA fragment showed much high homology with 5.8S and 25S rDNA region of *Candida neoformans*, a member of Basidiomycetous yeasts including *Phaffia rhodozyma*. The 730-bp nucleotide sequence of *Phaffia rhodozyma* rDNA gene was registered in GenBank on July 28, 1997, with accession NO. AF 016256.

Example 4: The construction of vector for transforming *Phaffia rhodozyma*

To construct vectors for transforming *Phaffia rhodozyma* efficiently, we exploited pTPL5 vector containing the mutated L41 gene of Example 2 and pTPR4 vector containing the rDNA fragment of Example 3 (see FIG 2). Particularly, we constructed pTPLR1 vector for transforming *Phaffia rhodozyma*, using the 3.7-kb fragment of pTPL5 as a cycloheximide-resistant marker and the 730-bp rDNA fragment of pTPR4 as a targeting sequence into *Phaffia rhodozyma* genome with multicopy. The 3.7-kb *Xba*I-*Sal*I fragment of pTPL5 containing the mutated L41 gene was treated with the Klenow enzyme and inserted into the *Bal*I site of pTPR4. The resulting plasmid, pTPLR1 (see FIG 3), was introduced into *E.*

coli DH5α strain, and the transformed *E. coli* strain was deposited in Korean Collection for Type Cultures (KCTC) on October 21, 1998 (accession NO: KCTC 0535BP).

We also constructed a plasmid, pTPLR2, which has the reverse direction of expressed sequence. The pTPLR1 and pTPLR2 vectors were digested with *Sma*I or *Bgl*I-*Kpn*I restriction enzymes, before the vector was brought to the transformation and integrated into the rDNA region of *Phaffia rhodozyma* genome.

Example 5: The transformation of *Phaffia rhodozyma* with pTPLR1 vector

To transform *Phaffia rhodozyma* with the pTPLR1 vector efficiently, we developed the transformation method, which is based upon the method for transforming a Basidiomycetous yeast, *Cryptococcus neoformans* (Varma *et al., Infect. Immun.*, 60, 1101, 1992) but optimized for *Phaffia rhodozyma*. Electroporation procedure was employed in the process of this invention. Particularly, *Phaffia rhodozyma* cells from a log-phase cluture in 50 ml of YM medium were harvested by centrifuge at 3,000 rpm for 10 minutes, then washed twice with equal volume of electroporation buffer (270 mM sucrose, 10 mM Tris, 1 mM $MgCl_2$, pH 8.0) containing 1 mM dithiothreitol (; DTT), and resuspended in the

electroporation buffer without DTT. The linearized plasmid pTPLR1 (200 ng) was mixed with a 50 μl aliquot (approximately 2×10^7 cells) of the cell suspension, and transferred to a cuvette (0.2-cm electrode gap; Bio-Rad, USA). We performed electroporation (Gene Pulser II; Bio-Rad, USA) under the various ranges of electric pulse (0.8 to 1.2 kV), internal resistance (400 to 800 Ω) and capacitance (25 to 50 μF). The electroporated cells were resuspended in 1 ml of YM medium and transferred to a test tube for incubation. After being shaken for 12 to 16 hours at 23°C, cells were spread on YM agar medium containing 10 μg/ml of cycloheximide and incubated at 23°C for 4 to 5 days.

Figure 4 shows the relationship between the condition of electroporation and the transformation efficiency or cell viability. The transformation efficiency was mainly dependent on the capacitance, preferably of 50 μF rather than 25 μF. In summary, more transformants were produced when an electric pulse of 0.8 kV was delivered and internal resistance of 600 Ω was set with a capacitance of 50 μF, generating pulse lengths of 18 to 20 ms. Under such condition, approximately 30% of cells survived, and transformation efficiencies of 800 to 1000 transformants per μg of DNA could be routinely obtained with pTPLR1 linearized either by *Sma*I or by *Bgl*I-*Kpn*I.

Using the optimized process, we transformed *Phaffia rhodozyma* with various vectors and observed the colony formation on the YM agar medium containing cycloheximide.

Interestingly, there was no transformant with pTPLR2 in any condition, suggesting that L41 gene is expressed only when the transcriptional direction of the integrated L41 gene is the same as that of rDNA.

Without the restriction of pTPLR1 before transformation, no colony was formed. This may result from the fact that rDNA does not have the autonomous replication sequence (; ARS) or its similar function.

A vector carrying cycloheximide-resistant L41 gene but not containing rDNA sequence, was introduced into *Phaffia rhodozyma*. In this case, a few colonies were observed. We suspected that the mutated L41 gene in the vector would replace endogenous L41 gene in the genome, rather than be integrated in directed position.

In addition, we transformed *Phaffia rhodozyma* with a vector in which the promoter of L41 gene was deleted, and observed transformed colonies. The Southern blot analysis of this transformant showed the same hybridization pattern as that of nontransformant control. This indicates that in this case also the transplacement has occurred, rather than be integrated in the directed position.

Example 7: Southern blot analysis of the transformants

To assess the stability of the introduced foreign DNA in *Phaffia rhodozyma* genome according to this invention, we performed Southern blot analysis of genomic DNA, which is prepared from pTPLR1 transformants or nontransformant control (see FIG 5). The genomic DNA was digested with *Sma*I or *Eco*RI enzyme, and the 2.2-kb *Sal*I fragment of pTPL2 was used as a probe in the hybridization. The intensity of colored band was measured by the scanning densitometer (Model GS-700 Imaging Densitometer, Bio-Rad, USA).

Southern blot analysis, in which genomic DNA of transformants was digested with *Sma*I, showed two colored bands at 9.0-kb and 4.1-kb. A signal at 9.0-kb is observed both in a nontransformant control and in the transformants, indicating that this band originated form the endogenous *Phaffia rhodozyma* L41 gene. A much stronger signal at 4.1-kb also was detected in transformants, but not in the control. This was expected from the restriction map of the transforming plasmid (see FIG 6). The size and relative intensity of signal at 4.1-kb suggested that multiple copies (approximately, 7 copies) of the transforming plasmid had been integrated.

In another Southern blot with *Eco*RI digestion, two bands at 5.8-kb and 2.8-kb were found only in

transformants (see FIG 5). The 5.8-kb band originated from a 3.2-kb rDNA fragment and a 2.6-kb L41 gene fragment, and the 2.8-kb band originated from a 1.7-kb rDNA fragment and a 1.1-kb L41 gene fragment. Integration probably occurs as diagrammed in Figure 6.

These results were reproducible in Southern blot with rDNA probe. Most importantly, copy number did not decrease after a prolonged cultivation in YM medium with or without cycloheximide, indicating that the transforming plasmid was integrated into the chromosome and maintained stably.

INDUSTRIAL APPLICABILITY

As shown above, the vectors for transforming *Phaffia rhodozyma* of the present invention comprises rDNA and cycloheximide-resistant L41 gene, which are useful for the stable integration of foreign DNA into host genome and for the convenient selection of transformants, respectively. These vectors are, therefore, applicable to the transformation of yeast cells including *Phaffia rhodozyma*, in combination with the transforming process of this invention, where yeast cells are transformed through the optimized electroporation.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is Claimed is

1. An L41 gene encoding a *Phaffia rhodozyma* ribosomal protein whose amino acid sequence is described by SEQ ID NO: 3.
2. The L41 gene of claim 1, wherein the genomic sequence of the gene is described by SEQ ID NO: 1.
3. The L41 gene of claim 1, wherein the cDNA sequence of the gene is described by SEQ ID NO: 2.
4. The L41 gene of claim 1, wherein the codons representing the amino acid sequence at position 56 is replaced by the codons representing glutamine.
5. A ribosomal DNA of *Phaffia rhodozyma*, which is described by SEQ ID NO: 4.
6. A vector for transforming *Phaffia rhodozyma*, comprising a cycloheximide-resistant gene and a portion of *Phaffia rhodozyma* ribosomal DNA.
7. The vector of claim 6, wherein the cycloheximide-resistant gene is the L41 gene of claim 4.
8. The vector of claim 6, wherein the *Phaffia rhodozyma* ribosomal DNA is the ribosomal DNA of claim 5.
9. The vector of claim 6, wherein the vector is pTPLR1 represented by figure 3.
10. A process of transforming yeast with the vector of claim 6.
11. The process of claim 10, the yeast is *Phaffia*

rhodozyma.

12. The process of claim 10, wherein the vector of claim 6 is cleaved into a linear form.

13. The process of claim 10, wherein the transformation is performed by electroporation under an electric pulse of 0.8~1.2 kV, an internal resistance of 400~800 Ω, and a capacitance of 25~50 μF.

FIG. 1

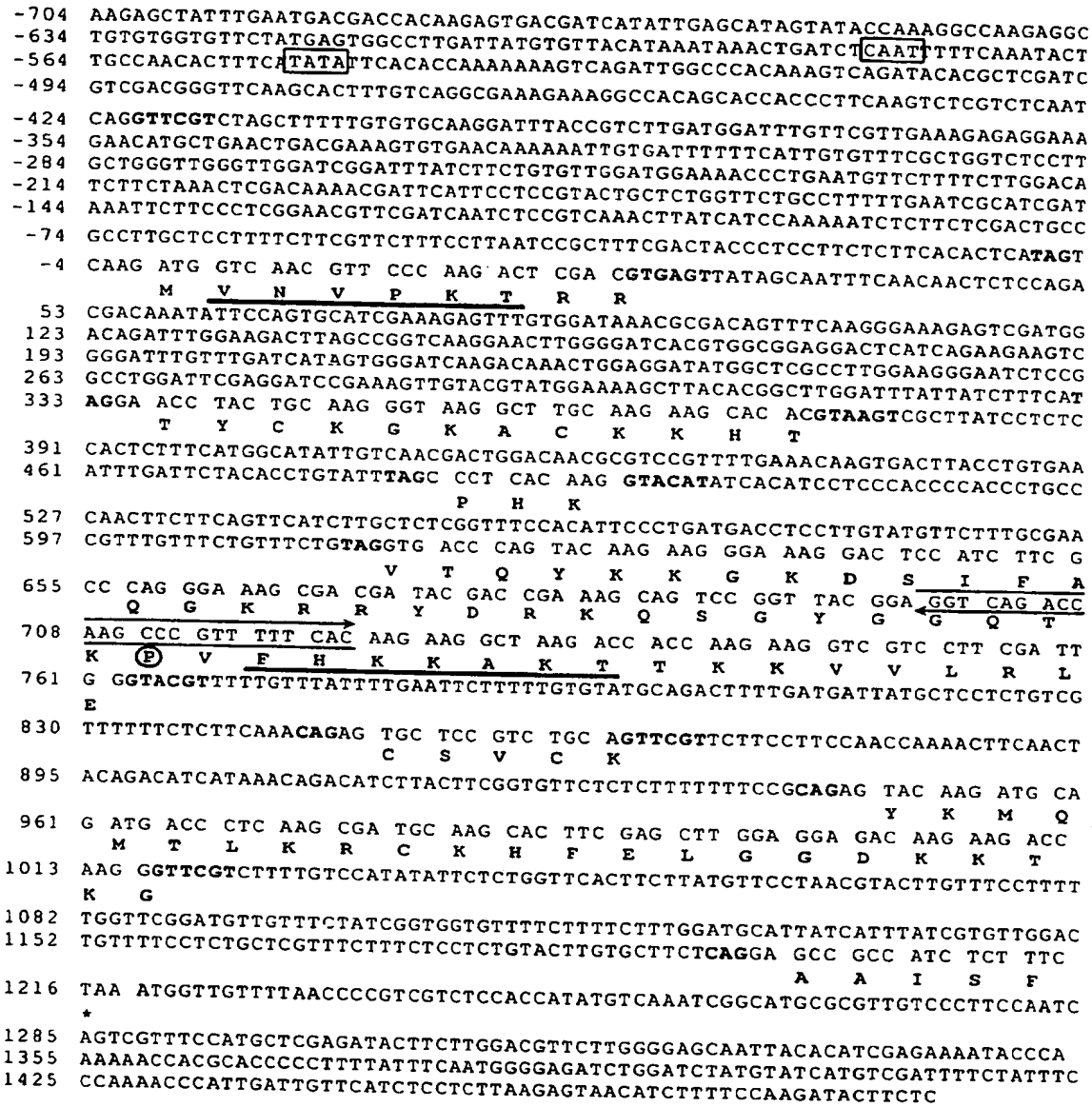
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FIG. 2

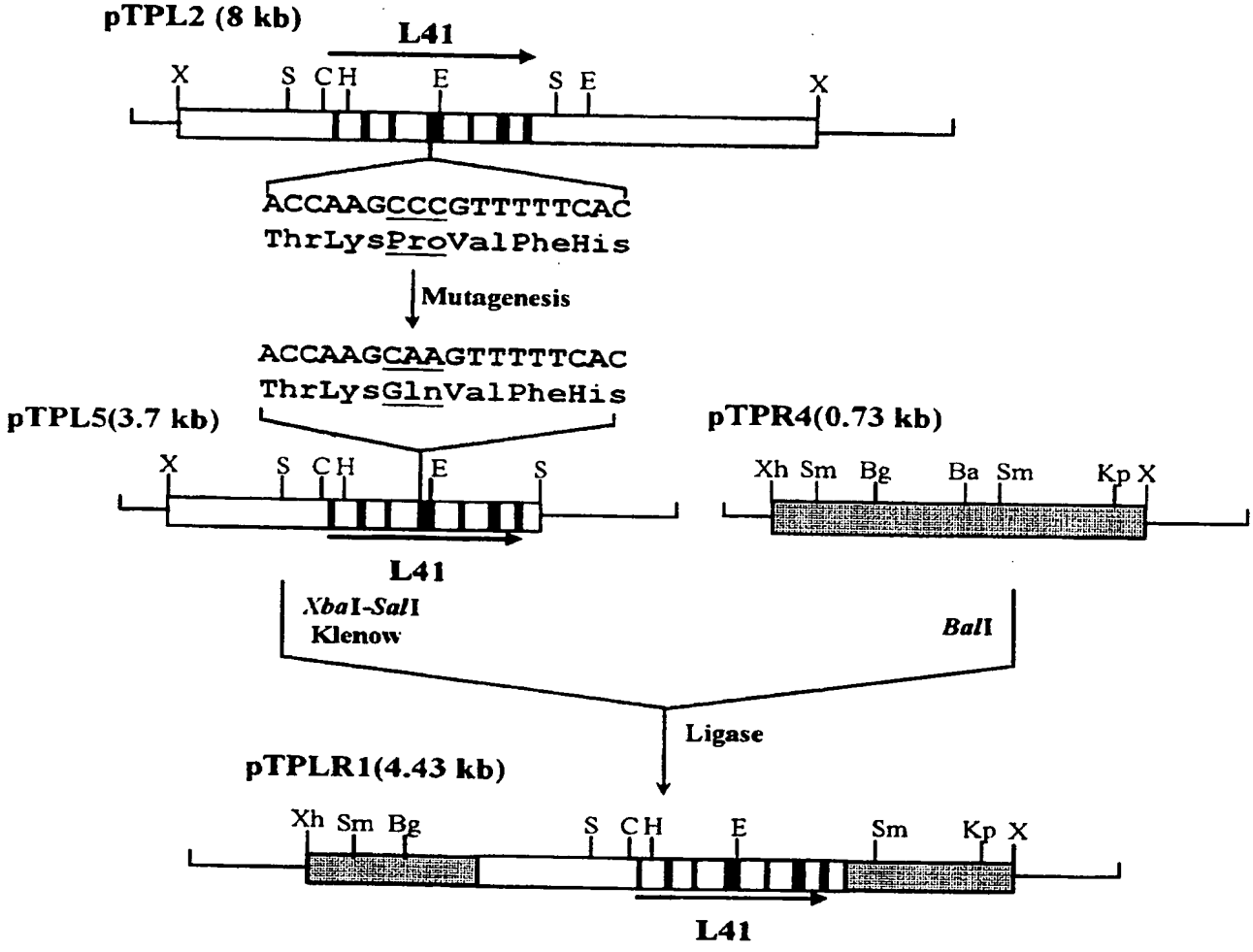
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FIG. 3

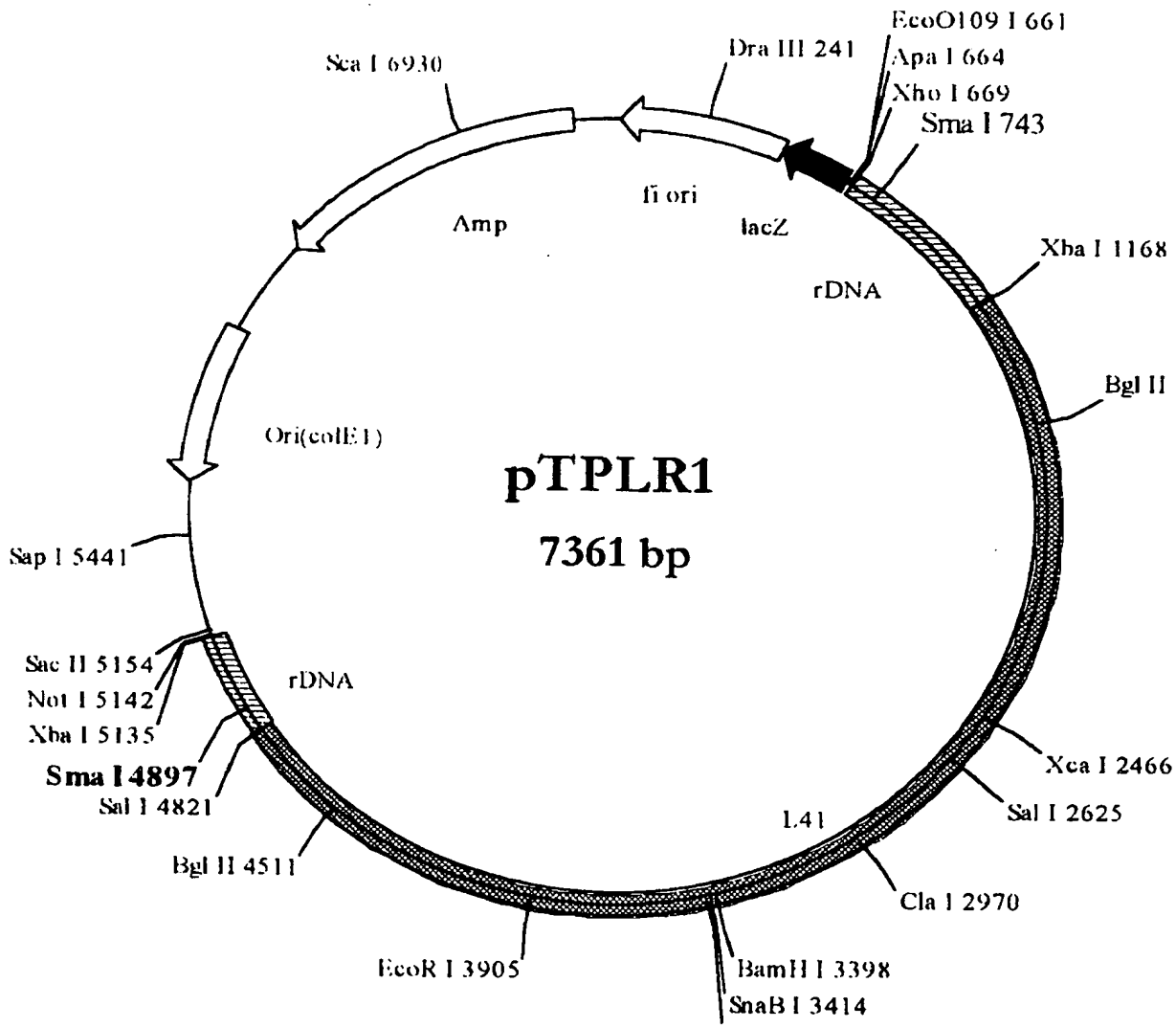
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FIG. 4

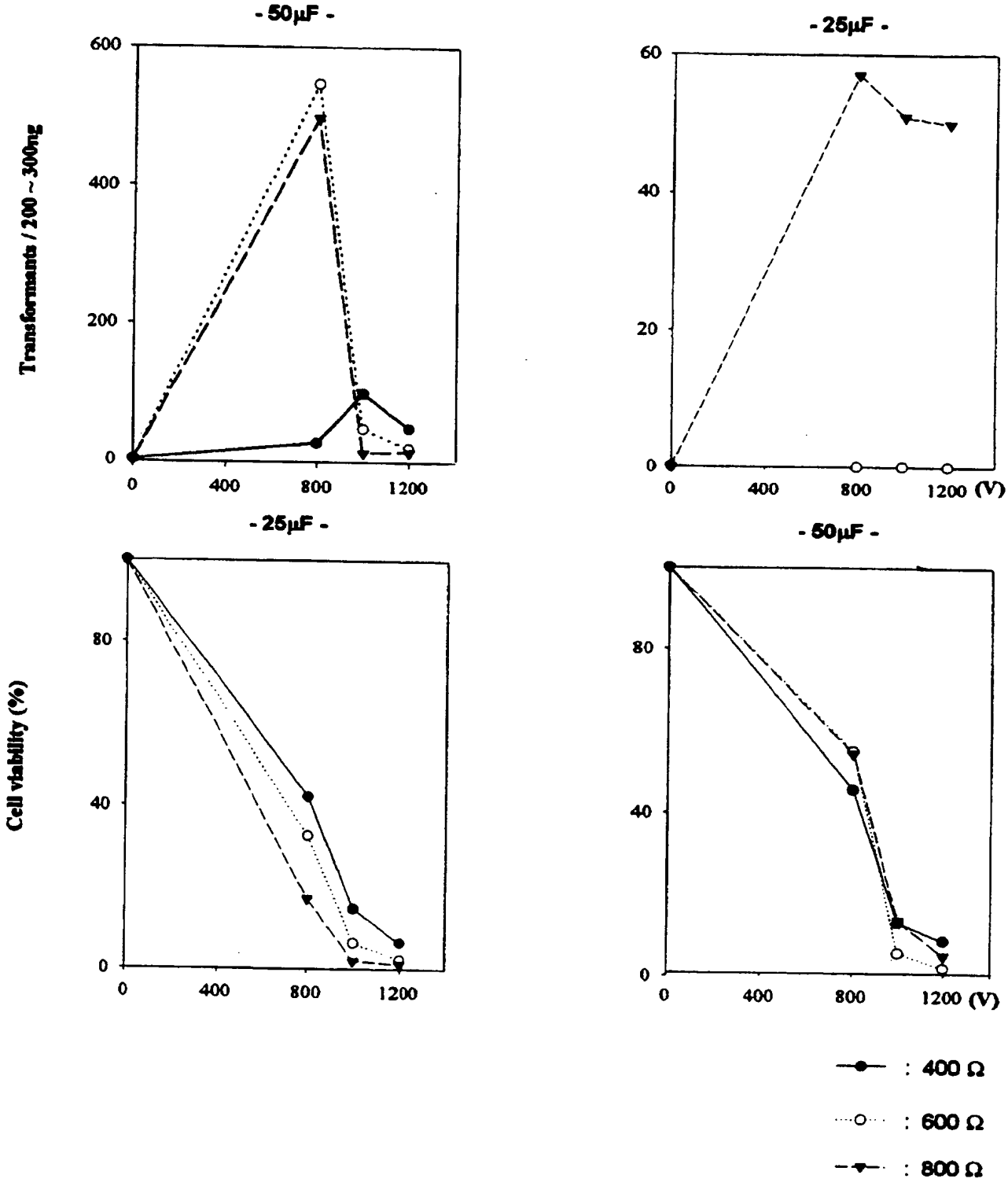
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FIG. 5

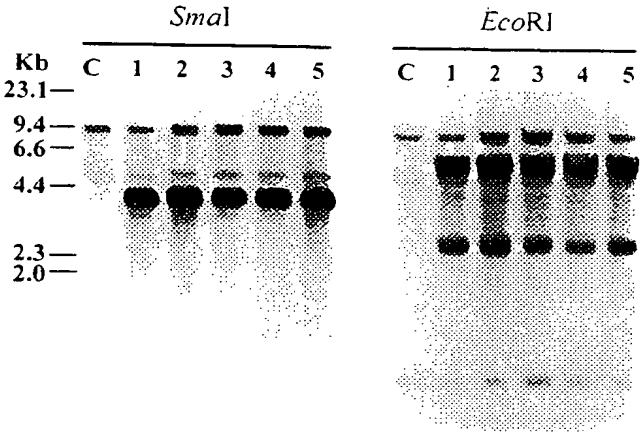
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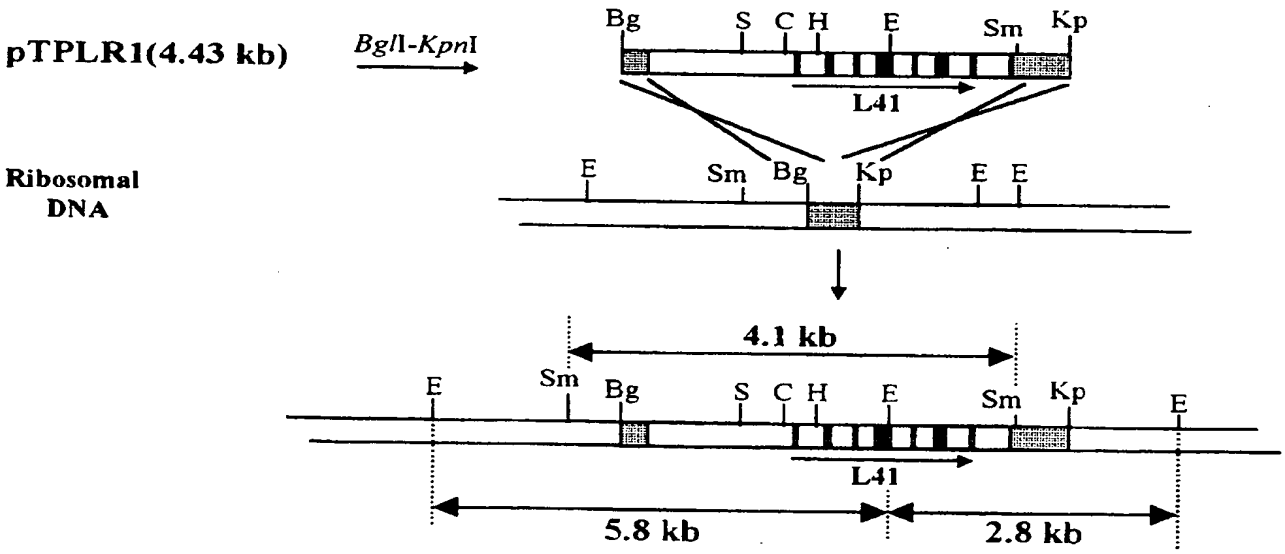
FIG. 6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Haitai Confectionery Co., Ltd.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00265

A. CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C 12 N 15/81; C 12 N 1/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C 12 N 15/81; C 12 N 1/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97/23 633 A1 (GISTBROCADES B.V.), 03 July 1997 (03.07.97), abstract; claims 1,4,6,7,9,10,12,14-18,23-25,28-31,34,38,40-47.	1-11
A	WO 94/06 918 A2 (GIST-BROCADES N.V.), 31 March 1994 (31.03.94), page 7, lines 20-33; examples 4,8,9; fig.4,5.	1-12
A	WERY J. et al.:"High copy number integration into the ribosomal DNA of the yeast Phaffia rhodozyma", Gene 1997, Vol.184, pages 89-97,90,91; abstract.	1,6,9,10-12
A	KAWAI et al.:"Drastic Alteration of Cycloheximide Sensitivity by Substitution of One Amino Acid of Yeasts", J.Bacteriol., January 1992, Vol.174, No.1, pages 254-262, totality.	1-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 September 1999 (02.09.99)	14 September 1999 (14.09.99)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/200	Authorized officer Mosser Telephone No. 1/53424/437

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 99/00265

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9723633	03-07-1997	AU A1 13087/97	17-07-1997
		CA AA 2241267	03-07-1997
		EP A1 870042	14-10-1998
		EP A1 780474	25-06-1997
WO A2 9406918	31-03-1994	AU A1 46242/93	17-03-1994
		AU B2 673847	28-11-1996
		CA AA 2105957	12-03-1994
		EP A1 590707	06-04-1994
		FI A0 933993	10-09-1993
		FI A 933993	12-03-1994
		JP T2 7501225	09-02-1995
		NO A0 933250	10-09-1993
		NO A 933250	14-03-1994
		NZ A 248628	27-02-1996
		US A 5840528	24-11-1998
		WO A3 9406918	07-07-1994
		EP X 586751	16-03-1994

COPY FOR IB

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Artcle 36 and Rule 70)

Applicant's or agent's file reference 9FPO-05-02	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. **PCT/KR99/00265**	International filing date *(day/month/year)* 29 MAY 1999 (29.05.1999)	Priority date *(day/month/year)* 31 OCTOBER 1998 (31.10.1998)

International Patent Classification (IPC) or national classification and IPC

IPC7 C12N 15/81, C12N 1/19

Applicant

KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also acconpanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demamd	Date of completion of this report
19 MAY 2000 (19.05.2000)	27 MARCH 2001 (27.03.2001)

Name and mailing address of the IPEA/KR
Korean Industrial Property Office
Government Complex-Taejon, Dunsan-dong, So-ku, Taejon
Metropolitan City 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
LIM, Hea Joon
Telephone No. 82-42-481-5590

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International aplication No.
PCT/KR99/00265

I. Basis of the report

1. With regard to the elements of the international application:*

[X] the international application as originally filed

[ ] the description:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

[ ] the claims:
pages _____ , as originally filed
pages _____ , as amended (together with any statment) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

[ ] the drawings:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

[ ] the sequence listing part of the description:
pages _____ , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application(under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide** and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained inthe international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disc losure in the international applicationas as filed has been furinshed.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages _____

[ ] the claims, Nos. _____

[ ] the drawings, sheet _____

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box(Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed." and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION

International aplication No.
PCT/KR99/00265

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-5, 6-9, 10-13	YES
	Claims		NO
Inventive step (IS)	Claims	1-5, 6-9, 10-13	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-5, 6-9, 10-13	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

1) The following document have been considered for the purpose of this report:
D1= J. bacteriol., 1992, vol. 174, No. 1, pp 254-262
D2=WO 94/06918 (GIST-BROCADES)
D3=Gene vol.184, pp89-97, 1997
D4=WO 97/23633 (GIST-BROCADES)

2) Novelty
Claims 1-5 relate to novel L41 gene encoding a Phaffia rhodozyma ribosomal protein , which can be mutated to acquire cycloheximide resistancy. Document D1 discloses the sequence of L41 gene of some strains of yeast including S. cerevisiae and C. maltosa. not the phaffia rhodozyma which is useful for the production of astaxanthin. Since the prior art does not provide the nucleotide sequence of ribosomal protein L41 of Phaffia rhodozyma, the claims 1-5 are considered to be novel.
Claims 6-9, 10-13 relate to a vector for transforming Phaffia rhodozyma comprising a cycloheximide-resistant gene utilizing L41 and ribosomal DNA, and the process of transforming yeast using same vector. Document D2, D3, and D4, the close prior arts in the present case, disclose the vector system comprising antibiotic resistance gene (such as kanamycin-resistant gene) as a selectable marker and ribosomal DNA from Phaffia rhodozyma for an integration site after transformation. Since claims 6-9, 10-13 utilize the L41 gene encoding a Phaffia rhodozyma ribosomal protein, which is mutated to acquire cycloheximide resistancy, different from prior arts utilizing antibiotic resistant genes came from microorganism, claims 6-9, 10-13 are considered to be novel.

3) Inventive Step
Claims 1-5 relate to novel L41 gene encoding a Phaffia rhodozyma ribosomal protein. Document D1 did not provide the nucleotide sequence of ribosomal protein L41 of Phaffia rhodozyma. Claims 6-9, 10-13 relate to a vector for transforming Phaffia rhodozyma comprising a cycloheximide-resistant gene utilizing L41 and ribosomal DNA, process of transforming yeast using same vector. Compare to vector systems in document D2, D3, and D4 comprising antibiotic resistance gene as a selectable marker and ribosomal DNA from Phaffia rhodozyma, it is certainly beneficial to the transforming yeast and maintaining the culture along with overproduction of desired product such as astaxanthin, utilizing its own mutated ribosomal protein L41 of Phaffia rhodozyma as a selectable marker in the case of claims 6-9, 10-13, through optimizing the conditions for growing even with overproduction of desired product. It is reported that yeast mutants transformed with bacterium selectable marker and ribosomal DNA overproducing desiredi product has problem of decreased growth rate. These problems were overcome in this invention. Therefore, the subject-matter of claim 6-9, 10-13 appear to involve an inventive step.

4) Industrial applicability
The subject matter of claim 1-5, 6-9, 10-13 is considered to be industrially applicable.

INTERNATIONAL PRELIMINARY EXAMINATION

International aplication No.
PCT/KR99/00265

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

Application No. Patent No.	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date (valid claim) *(day/month/year)*
PCT/NL93/00187	31.03. 94	10. 09. 93	11. 09. 92

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day/month/year)*	Date of written disclosure referring to non-written disclosure *(day/month/year)*

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 21 June 2000 (21.06.00)	
International application No. PCT/KR99/00265	**Applicant's or agent's file reference** 9fpo0502
International filing date (day/month/year) 29 May 1999 (29.05.99)	**Priority date** (day/month/year) 31 October 1998 (31.10.98)
Applicant CHOI, Eui-Sung et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

19 May 2000 (19.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin d s Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No.: (41-22) 338 83 38